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(54) Title: RECOMBINANT ANTIGEN FOR DIAGNOSING RHEUMATOID ARTHRITIS

(57) Abstract

A method is described for diagnosing rheumatoid arthritis by providing a recombinant antigen (RAMA) and detecting rheumatoid arthritis-associated IgM antibodies against the RAMA antigen in patient sera. The RAMA antigen comprises SEQ ID NO:3 and peptides substantially homologous thereto. A purified and isolated DNA encoding the RAMA antigen and a transformed host containing the DNA are also disclosed.

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## 5 RECOMBINANT ANTIGEN FOR DIAGNOSING RHEUMATOID ARTHRITIS

Background of the Invention

10 This invention relates to a method for diagnosing  
rheumatoid arthritis. More particularly, this invention  
relates to a method for objectively diagnosing  
rheumatoid arthritis by quantitative determination of  
the presence or absence of rheumatoid arthritis-  
associated antibodies in patient sera that react with a  
recombinant antigen. The invention also relates to the  
recombinant antigen and a molecular clone of the gene  
thereof.

15 Rheumatoid arthritis is a chronic systemic  
rheumatic disease that affects a significant percentage  
of the population. Traditionally, it has been diagnosed  
subjectively through clinical observation and dominant  
complaints by a patient. P. Lipsky, *Rheumatoid*  
20 *Arthritis*, in Harrison's Principles of Internal Medicine  
1423 (1987). Thus, clinical diagnosis of rheumatoid  
arthritis is subject to the skill of the diagnostician  
and the severity of disease symptoms in the patient.

25 For an objective diagnosis of rheumatoid arthritis,  
the presence of rheumatoid factor (Rf) in the serum of  
rheumatoid arthritis patients is routinely determined.  
Rf is an autoantibody that binds to the constant region  
of IgG immunoglobulins. The standard test for  
30 determining the presence of Rf in blood is an  
aggregation test wherein Rf causes aggregation of IgG.  
Rf has been detected in approximately 70% of patients  
exhibiting clinical symptoms of rheumatoid arthritis.  
These patients are thus termed "seropositive." The  
35 remaining 30% are classified as having "seronegative"  
rheumatoid arthritis. Numerous conditions besides  
rheumatoid arthritis are associated with the presence of  
rheumatoid factor. Therefore, the presence of Rf does  
not establish a conclusive diagnosis of rheumatoid  
40 arthritis. An objective method of diagnosing rheumatoid  
arthritis that is more closely correlated with clinical  
diagnoses than is the presence of Rf in sera is needed.  
Ideally, such an objective diagnostic test would be

5        quick and easy to perform and would not involve radioisotopes or be invasive to the patient.

         Sera from patients with various autoimmune rheumatic diseases contain circulating autoantibodies that are directed against cellular, mainly nuclear, components. E. Tan, 33 *Advances in Immunology* 167-240 (1982). These antibodies, designated as antinuclear antibodies (ANA), are specific for their respective autoimmune diseases and have been useful as diagnostic aids in clinical medicine. Some of the antigens against which these antibodies are directed have been produced by methods of biotechnology and used in diagnosis of respective autoimmune diseases. R. Michael & J. Keene, *Molecular Biology of Nuclear Autoantigen*, in 18 *Rheumatoid Disease Clinics of North America* 283-310 (D. Pisetsky, ed., 1992). Success in developing diagnostic tests against these autoimmune diseases suggests that a similar approach might be fruitful for rheumatoid arthritis.

         Sera from rheumatoid arthritis patients have also been found to contain antibodies to cellular components. A precipitin line forms in agar gel diffusion tests when sera from rheumatoid arthritis patients and extracts of certain Epstein-Barr virus-transformed human B lymphocyte cell lines, such as the WIL-2 and Raji cell lines, are placed in adjacent wells. M. Alspaugh & E. Tan, 19 *Arthritis and Rheumatism* 711-19 (1976). The antibody responsible for the precipitate is of the IgG type and the antigen against which it reacts is a nuclear antigen. Thus, the antigen is termed "rheumatoid arthritis nuclear antigen" or "RANA."

         Several problems would need to be overcome before a diagnostic test based on the presence of RANA could be developed. The identity of the antigen is not known. Even if it were known, it occurs in small quantities in cells and would be difficult to purify to homogeneity. Such purity is needed because false positives might

5 result if contaminants were copurified with the RANA, given the extreme sensitivity of serological tests that can be devised to detect small quantities of antigen.

For these reasons, the present invention discloses a different approach to quantitative detection of  
10 rheumatoid arthritis. This approach involves production of a recombinant antigen by recombinant DNA technology and detection of rheumatoid arthritis-associated antibodies to this novel antigen in patient sera. This recombinant antigen does not react with commercial anti-  
15 RANA antibodies.

#### Objects and Summary of the Invention

It is an object of the present invention to provide a method for diagnosing rheumatoid arthritis.

20 It is another object of the invention to provide a method for diagnosing rheumatoid arthritis by serological analysis of patient sera, such as by ELISA analysis.

It is also an object of the invention to provide a  
25 nucleic acid capable of directing expression of a recombinant antigen detectable by rheumatoid arthritis-associated antibodies.

It is still another object of the invention to provide a recombinant antigen detectable by rheumatoid  
30 arthritis-associated antibodies.

These and other objects may be accomplished by providing an antigen for diagnosing rheumatoid arthritis comprising a peptide having an amino acid sequence selected from the group consisting of SEQ ID NO:3 and  
35 sequences substantially homologous thereto, wherein the antigen is reactive with rheumatoid arthritis-associated antibodies. The antigen can be expressed in prokaryotic or eukaryotic host cells or can be synthesized chemically. The rheumatoid arthritis-associated  
40 antibodies are of the IgM subtype.

5           The invention also comprises a purified and isolated DNA for use in securing expression in a host cell of a peptide having at least a part of the primary structural conformation and the antigenic activity of naturally-occurring RAMA protein; the DNA selected from  
10 the group consisting of:

(a) SEQ ID NO:2;

(b) DNA that hybridizes to SEQ ID NO:2 or fragments thereof; and

(c) DNA that, but for the degeneracy of the  
15 genetic code, would hybridize to the DNA defined in (a) and (b). The purified and isolated DNA can further comprise a vector adapted for transformation of a host, wherein the vector is selected from the group consisting of plasmids, cosmids, phagemids, phages, viruses, and  
20 the like. The host can be a prokaryotic cell, such as *E. coli*, or a eukaryotic cell.

#### Brief Description of the Drawings

FIG. 1 is a graph depicting the results of ELISA  
25 tests of sera from healthy (H), rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) sera according the present invention.

#### Detailed Description

30           Before the present recombinant antigen and molecularly cloned gene thereof are disclosed and described, it is to be understood that this invention is not limited to the particular process steps and materials disclosed herein as such process steps and  
35 materials may vary somewhat. It is also to be understood that the terminology used herein is used for the purpose of describing particular embodiments only and is not intended to be limiting since the scope of the present invention will be limited only by the  
40 appended claims and equivalents thereof.

5           It must be noted that, as used in this  
specification and the appended claims, the singular  
forms "a," "an," and "the" include plural referents  
unless the content clearly dictates otherwise. Thus,  
for example, reference to an antigen containing "a  
10 peptide" includes a mixture of two or more peptides,  
reference to "a host cell" includes reference to one or  
more of such host cells, and reference to "a plasmid"  
includes reference to a mixture of two or more plasmids.

15           In describing and claiming the present invention,  
the following terminology will be used in accordance  
with the definitions set out below.

          As used herein, "RAMA" means the Rheumatoid  
Arthritis IgM-associated Antigen of the instant  
invention, encoded by the plasmid deposited as ATCC  
20 69605.

          As used herein, "peptide" means peptides of any  
length and includes proteins. The terms "polypeptide"  
and "oligopeptide" are used herein without any  
particular intended size limitation, unless a particular  
25 size is otherwise stated.

          As used herein, "DNA" means DNA and other nucleic  
acids capable of storing genetic information. For  
example, an RNA produced by *in vitro* transcription of a  
RAMA gene is included within the scope of the term DNA.

30           As used herein, "vector" means any genetic element  
capable of replicating in a host cell and of carrying  
foreign nucleic acid that is inserted into the vector.  
Illustrative of vectors that can be used within the  
scope of the invention are plasmids, cosmids, phagemids,  
35 phages, viruses, and the like.

          As used herein, "substantially homologous" refers  
to polynucleotides and polypeptides that retain  
functionality despite differences in primary structure  
from polynucleotides and polypeptides to which they are  
40 compared. For example, a polynucleotide substantially  
homologous to SEQ ID NO:2 is one that can secure

5 expression in a host cell of a polypeptide product  
having at least a part of the primary structural  
conformation and the antigenic activity of the  
naturally-occurring protein having the amino acid  
sequence of SEQ ID NO:3, the polynucleotide selected  
10 from (a) polynucleotides that hybridize to SEQ ID NO:2  
or fragments thereof and (b) polynucleotides that, but  
for the degeneracy of the genetic code, would hybridize  
to the polynucleotides defined in SEQ ID NO:2 and (a).  
By way of further example, a polypeptide substantially  
15 homologous to SEQ ID NO:3 is one that retains  
functionality as an antigen reactive with rheumatoid  
arthritis-associated antibodies although it may include  
additional amino acid residues or be a truncation,  
deletion variant, or substitution variant of SEQ ID  
20 NO:3. A substitution variant is one that contains a  
conservative substitution of one or more amino acid  
residues. A conservative substitution is a substitution  
of one amino acid residue for another wherein  
functionality of the peptide is retained, in this case,  
25 functionality as an antigen reactive with rheumatoid  
arthritis-associated antibodies. Amino acid residues  
belonging to certain conservative substitution groups  
can sometimes substitute for another amino acid residue  
in the same group. One such grouping is as follows:  
30 Pro; Ala, Gly; Ser, Thr; Asn, Gln; Asp, Glu; His; Lys,  
Arg; Cys; Ile, Leu, Met, Val; and Phe, Trp, Tyr. M.  
Jimenez-Montano & L. Zamora-Cortina, Evolutionary model  
for the generation of amino acid sequences and its  
application to the study of mammal alpha-hemoglobin  
35 chains, Proc. VIIth Int'l Biophysics Congress, Mexico  
City (1981). Other variations that are to be considered  
substantially homologous include substitution of D-amino  
acids for the naturally occurring L-amino acids,  
substitution of amino acid derivatives such as those  
40 containing additional side chains, and substitution of  
non-standard amino acids, i.e.  $\alpha$ -amino acids that are



5 rare or do not occur in proteins. The primary structure of a substantially homologous polypeptide is limited only by functionality.

A gene encoding a novel antigen ("RAMA") was molecularly cloned and expressed in bacterial and eukaryotic protein expression systems as described in  
10 copending U.S. Patent Application Serial No. 08/019,780 filed February 19, 1993, hereby incorporated by reference. Briefly, the steps involved in cloning and expressing the RAMA antigen are as follows.  
15 Polyadenylated mRNA was isolated from about  $1 \times 10^8$  human Raji cells (ATCC no. CCL 86) using the "FAST TRACK" mRNA isolation kit (Invitrogen, San Diego, California). The cells were lysed, homogenized, incubated with protease, and then subjected to oligo(dT)cellulose chromatography.  
20 The resulting polyadenylated RNA was then used as template material to prepare double-stranded cDNA using a commercial kit ( $\lambda$  Librarian, Invitrogen). The method used in this kit is the method described by Okayama and Berg, 2 Molecular and Cellular Biology 161 (1982), and  
25 Gubler and Hoffman, 25 Gene 263 (1983). The ends of the cDNA were made blunt-ended by treatment with T4 polymerase. EcoRI linkers were joined to the blunt-ended cDNA by T4 DNA ligase. The linkers had the following sequence:

30 5'-AATTCGCGGCCGC-3' (SEQ ID NO:1)  
3'-GCGCCGGCG-5'

The 5' end of the shorter oligomer comprising the linker was phosphorylated whereas the 5' end of the longer oligomer (SEQ ID NO:1) was not. Once the linkers had  
35 been added, the cDNA was treated with T4 polynucleotide kinase to phosphorylate the protruding 5' end of the EcoRI linker. The double-stranded cDNA resulting from these procedures included a distribution of various lengths of cDNA as well as excess unreacted linkers.  
40 The unreacted linkers were removed and cDNA in the range of 1-5 kbp was selected by fractionating the cDNA by electrophoresis in an agarose gel. After fractionation

5 was complete, the gel was removed from the gel apparatus, the cDNA was visualized with ethidium bromide, and slices of the cDNA lane were cut corresponding to the desired size of 1-5 kbp. The cDNA was immediately electroeluted.

10 The size-selected double-stranded cDNA was then cloned in the phage  $\lambda$ gt11 cloning vector. R. Young & R. Davis, 80 Proc. Nat'l Acad. Sci USA 1194-98 (1983); T. Hyynh et al., in 1 *DNA Cloning: A Practical Approach* 49-78 (D. Glover, ed, IRL Press, Oxford, 1985). The *EcoRI* cloning site in this vector is located within the *E. coli lacZ* gene that was inserted into the phage  $\lambda$  DNA in making the  $\lambda$ gt11 vector. The *lacZ* gene codes for the enzyme  $\beta$ -galactosidase. DNA fragments inserted into this gene by cloning at the *EcoRI* site result in fusion genes that make an inactive recombinant  $\beta$ -galactosidase enzyme under the control of the *lac* promoter. Recombinant phage can be recognized and selected by their inability to form blue-colored plaques on indicator plates containing the lactose analog 5-bromo-25 4-chloro-3-indolyl- $\beta$ -D-galactoside (X-gal). Lambda gt11 phage are *lac*<sup>+</sup> and thus able to cleave colorless X-gal into metabolites that self-assemble into a blue-colored indole compound. *EcoRI*-digested, dephosphorylated  $\lambda$ gt11 DNA was obtained from Invitrogen.

30 Ligated DNA was then packaged in the "PACKAGENE" phage  $\lambda$  packaging system obtained commercially from Promega Corp. (Madison, Wisconsin), and the titer of recombinant phage was determined according to the supplier's instructions.

35 Recombinant antigen was isolated using a nonradioactive immunoblotting technique described in the technical manual for the "PROTOBLOT" Immunoscreening System from Promega Corp. Y1090 host cells were infected with  $3 \times 10^4$  plaque forming units (PFU) of recombinant phage from the  $\lambda$ gt11 library and then plated 40 on agar plates. The plates were overlaid with dry

5 nitrocellulose filters previously saturated with 10 mM  
IPTG and incubated at 37° C. During incubation, phage  
and proteins released from lytically-infected cells  
adhered to the filters. The filters were removed from  
the plates and then blocked to prevent other proteins  
10 from adhering to the plates. Serum (diluted 1:20 with  
TBST buffer: 10 mM Tris HCl, pH 8.0, 1 mM EDTA, 0.05%  
"TWEEN-20") from a patient clinically determined to have  
rheumatoid arthritis was then incubated with the filter.  
Then, the filter was washed in TBST to remove antibodies  
15 that were bound nonspecifically. Then the filter was  
incubated with an anti-IgM antibody-alkaline phosphatase  
conjugate (Kirkegaard & Perry Laboratories, Inc.,  
Gaithersburg, MD; diluted 1:100 with TBST). The filter  
was then washed again, and the color development  
20 substrates, nitro blue tetrazolium (NBT) and 5-bromo-4-  
chloro-3-indolyl phosphate (BCIP), were added. Positive  
plaques produced a dark purple color as a result of  
alkaline phosphatase activity. Positive plaques were  
retested and purified by replating until all of the  
25 plaques on a test plate yielded a positive signal.

A lysogen of a purified positive recombinant  $\lambda$ gt11  
phage was generated according to Technical Bulletin No.  
006 of Promega Corp. Recombinant phage DNA was isolated  
from the  $\lambda$ gt11 lysogen, using an alkaline lysis miniprep  
30 protocol described in T. Maniatis et al., *Molecular  
Cloning: A Laboratory Manual* (Cold Spring Harbor  
Laboratories, Cold Spring Harbor, New York, 1982). The  
DNA was digested with EcoRI, and the resulting DNA  
fragments were electrophoretically fractionated in a  
35 0.7% low melting agarose gel. Upon ethidium bromide  
staining and ultraviolet illumination, a unique 2600 bp  
band was revealed. This band was sliced from the gel,  
and the agarose was melted at 70°C. The DNA was then  
phenol extracted and precipitated with alcohol.

40 The 2600 bp EcoRI fragment was then recloned, using  
standard procedures, e.g. J. Sambrook et al., *Molecular*

5        *Cloning: A Laboratory Manual* (2d ed., 1989); T. Maniatis  
et al., *Molecular Cloning: A Laboratory Manual* (1982);  
F. Ausubel et al., *Current Protocols in Molecular*  
Biology (1987), at the *EcoRI* site of the plasmid  
expression vector "pTrcHis C," obtained from Invitrogen.  
10       This vector has the same reading frame as  $\lambda$ gt11,  
contains all the DNA sequences to obtain high level  
protein expression in *E. coli*, and also contains a  
sequence encoding 6 consecutive histidine residues,  
which allow the expressed protein to bind Ni-charged  
15       "PROBOND" resin (Invitrogen) so that the recombinant  
protein can be easily purified in a one-step procedure.  
The pTrcHis C plasmid containing the 2600 bp fragment  
was transformed into *E. coli* strain Top10 (obtained from  
Invitrogen).

20       Expression of the recombinant protein was  
demonstrated by Western blot analysis. Transformants  
were grown in Luria Broth (LB) at 37° C to an OD<sub>600</sub> of  
0.5. Then isopropylthio- $\beta$ -D-galactoside (IPTG), a  
gratuitous inducer of the *lac* operon, was added to a  
25       final concentration of 1 mM to induce expression of the  
recombinant protein. The transformants were grown an  
additional 3 hours at 30° C after induction. Then about  
200  $\mu$ l of culture was placed in a microfuge tube and  
centrifuged briefly to pellet the cells. The broth was  
30       removed and discarded and the pellet was resuspended in  
SDS-containing buffer. T. Maniatis et al., *supra*. The  
samples were heated for 2 minutes in a boiling water  
bath and loaded on a 10% SDS-polyacrylamide gel and  
electrophoresed overnight at 70 volts. T. Maniatis et  
35       al., *supra*. The proteins were transferred  
electrophoretically to a nitrocellulose membrane using  
the "POLYBLOT" Electrotransfer System according to the  
instruction manual (American Bionetics, Inc., Hayward,  
California). After transfer was complete, the membrane  
40       was removed and then blocked to prevent nonspecific  
binding of proteins. Serum (diluted 1:21) from a

5 patient with rheumatoid arthritis was added to the membrane and incubated for 1 hour. The membrane was then washed in TBST. Then the membrane was incubated with anti-IgM antibody-alkaline phosphatase conjugate (Kirkegaard & Perry), as in the plaque screening  
10 procedure. The membrane was then washed in TBST, and color was developed by addition of NBT and BCIP.

These tests revealed a single band corresponding to a protein of about 48 kD that reacted with the reference serum. About 4 kD of the protein sequence is derived  
15 from the plasmid vector, suggesting that the remaining 44 kD of protein produced by the expression vector is from the antigen that reacts with serum from a rheumatoid arthritis patient.

#### Sequencing of the Recombinant Antigen Gene

20 The cloned cDNA was subjected to nucleotide sequence analysis according to the method of F. Sanger et al., *DNA Sequencing with Chain-Terminating Inhibitors*, 74 Proc. Nat'l Acad. Sci. USA 5463 (1977). An open reading frame comprising a 993 bp segment of DNA  
25 was revealed. This open reading frame (SEQ ID NO:2) encodes a 331 amino acid protein (SEQ ID NO:3) comprising the recombinant RAMA antigen of the instant invention.

#### Purification of Recombinant Antigen from Bacteria

30 The recombinant RAMA protein expressed by the bacterial plasmid expression vector was purified using an Invitrogen "PROBOND" column according to the instructions supplied with the column. About 1 liter of LB also containing glucose and 50 µg/ml ampicillin was  
35 inoculated with 10 ml of an overnight culture of BL21 cells (F' ompT hsdS<sub>B</sub> [r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup> dcm]) (a protease<sup>-</sup> strain, Novagen, Madison, Wisconsin) containing the expression plasmid. The cells were grown for 2.5 hours, at which time IPTG was added to a final concentration of 1 mM to  
40 induce expression of the recombinant RAMA protein. The cells were incubated an additional 3 hours at 37° C

5 after induction. Then the cells were harvested by centrifugation, resuspended, and lysed with lysozyme and sonication. The cells were then centrifuged at 10,000 rpm. The recombinant RAMA protein was soluble and remained in the supernatant.

10 Expression of the recombinant RAMA protein was confirmed by Western Blot analysis. After electrophoresis of a sample of recombinant RAMA protein in polyacrylamide gel and electrophoretic transfer to a nitrocellulose membrane, nonspecific binding of protein  
15 was blocked. Serum from a rheumatoid arthritis patient was added to the membrane-bound protein at a dilution of 1:21 and incubated for 1 hour. The membrane was then washed and incubated with anti-human IgM-alkaline phosphatase conjugate. The membrane was again washed  
20 before color development substrate solution was added. A single protein band with an  $M_r$  of about 41,000 reacted with the serum from the rheumatoid arthritis patient. This is in reasonably good agreement with the predicted size of the RAMA protein (about 34 kd) based on the  
25 sequence.

#### Expression of Recombinant RAMA in Eukaryotic Cells

The 2600 bp DNA fragment containing the RAMA gene was recloned in the pBlueBachHis C baculovirus vector (Invitrogen) by standard methods. This pBlueBachHis C  
30 vector containing the RAMA gene was co-transfected with "BACULOGOLD" (Pharmingen, San Diego, California) baculovirus DNA into *Spodoptera frugiperda* Sf9 cells. Homologous recombination between these DNAs resulted in a recombinant virus with the RAMA gene expressed under  
35 the control of the viral polyhedrin enhancer/promoter elements. The recombinant virus was produced in Sf9 insect cells and purified as described in the Invitrogen manual. The virus stock was then used to prepare 10-fold dilutions for plaque purification of recombinant  
40 virus according to the Invitrogen manual.

5           Expression of the RAMA gene in pBlueBacHis C was confirmed by Western Blot analysis. About 1 ml of Sf9 insect cells infected 3 days earlier with virus containing the recombinant plasmid were pelleted and dissolved in 100  $\mu$ l of Laemmli buffer. U. Laemmli, 227  
10   Nature 680-85 (1970). The sample was boiled for 2 minutes and then loaded on a 7.5% SDS-polyacrylamide gel and electrophoresed overnight at 70 volts, as described above. The protein was transferred electrophoretically to a nitrocellulose membrane and nonspecific binding of  
15   protein was blocked, as described above. Serum from a rheumatoid arthritis patient was added to the membrane-bound protein at a dilution of 1:21 and incubated for 1 hour. The membrane was then washed with TBST and incubated with anti-human IgM-alkaline phosphatase  
20   conjugate for 30 minutes. The membrane was again washed with TBST before color development substrate solution was added. A single protein band with an  $M_r$  of about 100,000 reacted with the serum from the rheumatoid arthritis patient. The difference in  $M_r$ 's of the RAMA  
25   protein expressed in bacteria and in eukaryotic cells is believed due to glycosylation and perhaps other modifications of the expressed protein in eukaryotic cells. Recombinant RAMA protein produced by expression in this eukaryotic cell system was purified on a Ni-charged "PROBOND" column as described above. About 1.5  
30   mg of protein was purified from 50 ml of culture.

#### ELISA Test of the Recombinant RAMA Protein

          About 100  $\mu$ l of recombinant RAMA protein solution (1  $\mu$ g/ml of purified recombinant protein in PBS buffer, pH 7.4), produced by expression in the *E. coli* system and purified on a "PROBOND" column, was placed in a well  
35   of a polystyrene microtiter plate (High binding 96 well Corning plate) and incubated overnight at 4°C. The plate was washed and then blocked overnight at 4°C to prevent nonspecific binding. A 100  $\mu$ l aliquot of serum  
40   diluted 1:21 was added to the well and incubated for 1

5 hour, and then the well was washed. A 100  $\mu$ l aliquot of  
alkaline phosphatase-conjugated anti-human IgM  
(Kirkegaard & Perry) was added to the well and incubated  
for 1 hour, and then the well was washed again. Then  
100  $\mu$ l of alkaline phosphatase substrate, prepared by  
10 adding 5 mg of p-nitrophenolphosphate and 1 ml of 5X  
diethanolamine buffer (supplied by Kirkegaard and Perry)  
to 4 ml of distilled water, was added to the well and  
incubated at 37° C for 15 minutes. Then, the optical  
density was measured at 405 nm.

15 Sera from 60 patients with clinical symptoms of  
rheumatoid arthritis (35 were seropositive and 25 were  
seronegative for Rf), 20 individuals seropositive for an  
anti-DNA disease marker for SLE, and 20 healthy  
individuals were tested by the method outlined above.  
20 The results of these tests are summarized in FIG. 1 and  
the following table.

Serum	Total	RAMA <sup>+</sup>	RAMA <sup>-</sup>	Percent
Rf <sup>+</sup>	35	34	1	97
25 Rf <sup>-</sup>	25	11	14	44
Anti-DNA <sup>+</sup>	20	3	17	15
Healthy	20	0	20	0

30 Serum from all of the healthy subjects showed ELISA  
values below 0.250. Thus, a reading of 0.250 was taken  
as the cut-off value to determine a positive reaction.  
Of the 35 sera from seropositive rheumatoid arthritis  
patients, 34, or 97%, showed ELISA values above 0.250  
and, thus, were deemed to give a positive reaction. Of  
35 the 25 sera from seronegative rheumatoid arthritis  
patients, 11, or 44%, showed ELISA values above 0.250  
and, thus, were deemed to give a positive reaction.  
Three of the 20 Anti-DNA<sup>+</sup> control sera also gave positive  
reactions. Therefore, these results show that almost  
40 all seropositive rheumatoid arthritis patients could be  
diagnosed with the aid of this ELISA test to detect the



5        présence of antibodies in the serum against the RAMA  
recombinant antigen. Further, almost half of  
seronegative rheumatoid arthritis patients could be  
diagnosed as well. These results suggest that about 85%  
10        of rheumatoid arthritis cases could be diagnosed using  
this invention as compared to only about 70% using the  
standard Rf test.

Additional tests were conducted to demonstrate that  
RAMA is not Rf. An independent reference laboratory was  
contracted to conduct the standard Rf aggregation test  
15        using the recombinant RAMA protein. No aggregates of  
IgG were formed. This is a negative result, inasmuch as  
aggregates did form when Rf was assayed in the same  
manner as a positive control. Further, recombinant RAMA  
antigen was attached to the wells of a microtiter plate,  
20        and the bound RAMA antigen was then exposed to an  
enzyme-conjugated IgG antibody. A colorimetric assay of  
enzyme activity was conducted as described above. No  
enzyme activity was detected, indicating that the IgG  
antibody failed to bind to the RAMA protein. Finally,  
25        7 clinically normal subjects exhibiting a positive  
result when tested by ELISA for reaction with Rf, i.e.  
all 7 subjects were seropositive for Rf, were tested by  
ELISA with RAMA as the primary antigen. All 7 were  
seronegative for reaction with RAMA. These results  
30        demonstrate that the recombinant RAMA antigen that is  
the subject of the invention is not Rf.

#### Peptides with RAMA Activity

The scope of the present invention includes any  
peptide having the activity of a RAMA peptide. Such a  
35        peptide can include recombinant RAMA as in SEQ ID NO:3,  
and peptides substantially homologous thereto. An  
example of a peptide substantially homologous to the  
naturally-occurring RAMA is the recombinant RAMA  
described above, wherein 6 histidine residues were added  
40        to facilitate purification of the protein by affinity  
chromatography using a metal-containing resin. Despite

5 the addition of the 6 histidine residues, the  
recombinant RAMA was reactive with the rheumatoid  
arthritis-associated IgM antibodies. Peptides that are  
substantially homologous to RAMA can be synthesized by  
expression in host cells, as exemplified above, or by  
10 chemical synthesis.

Short peptides for detecting rheumatoid arthritis-  
associated antibodies can be identified and prepared as  
follows. Endoproteinase-lys.C (Boehringer Mannheim) is  
used according the supplier's directions to digest the  
15 RAMA protein into peptide fragments. These fragments  
are fractionated by HPLC and sequenced according to the  
method of N. Legendre & P.T. Matsudaira, *Gel  
Electrophoresis, in A Practical Guide to Protein and  
Peptide Purification for Microsequencing* 52-66 (P.T.  
20 Matsudaira, ed., 1989). Additional fragments are  
prepared by proteinase digestion of RAMA and separation  
on polyacrylamide gels. J. Sambrook et al., *Molecular  
Cloning: A Laboratory Manual* (2d ed., 1989). These  
fragments are subjected to Western blotting, H. Towbin  
25 et al., *Electrophoretic Transfer of Proteins from  
Polyacrylamide Gels to Nitrocellulose Sheets: Procedure  
and Some Applications*, 76 *Proc. Nat'l Acad. Sci. USA*  
4350 (1979), with identification of fragments bound by  
rheumatoid arthritis-associated antibodies. Those  
30 peptides reacting with the antibodies are sequenced.  
Following identification of the fragment or fragments of  
RAMA having epitopes recognized by the rheumatoid  
arthritis-associated antibodies, the process of  
digestion with a proteinase, Western blotting, and  
35 sequencing is repeated using a different proteinase to  
yield smaller peptides. This procedure leads to  
identification of a sequence recognized by the  
antibodies. From these data, oligopeptides with similar  
sequence are synthesized by chemical synthesis, B.  
40 Merrifield, 85 *J. Am. Chem. Soc.* 2149-2156 (1963); B.  
Merrifield et al., 21 *Biochemistry* 5020-31 (1982);

5 Houghten, 82 Proc. Nat'l Acad. Sci. USA 5131-35 (1985),  
hereby incorporated by reference, or biotechnological  
methods, J. Sambrook et al., *Molecular Cloning: A*  
10 *Laboratory Manual* (2d ed., 1989), and tested for  
reactivity to the rheumatoid arthritis-associated  
antibodies. Several peptidomimetic inhibitors of  
enzymes have been described using these techniques. A.  
Smith et al., *Design and Synthesis of Peptidomimetic*  
15 *Inhibitors of HIV-1 Protease and Renin: Evidence for*  
*Improved Transport*, 37 J. Med. Chem. 215 (1994); S.  
Francis et al., *Molecular Characterization and*  
*Inhibition of a Plasmodium falciparum Aspartic*  
*Hemoglobinase*, 13 EMBO J. 306 (1994); A. Garcia et al.,  
20 *Peptidomimetic Inhibitors of Ras Farnesylation and*  
*Function in Whole Cells*, 268 J. Biol. Chem. 18415  
(1993).

Deposit of Biological Material

A deposit of an *E. coli* strain containing a plasmid  
bearing a gene encoding the recombinant RAMA antigen  
described herein and used for diagnosing rheumatoid  
25 arthritis was deposited on April 13, 1994, with the  
following International Depository Authority: American  
Type Culture Collection, 12301 Parklawn Drive,  
Rockville, MD 20852 USA. The accession number of the  
deposited strain is ATCC 69605.

18

5. Sequence Listing

## (1) GENERAL INFORMATION:

10

(i) APPLICANT: Ramesh K. Prakash

15

(ii) TITLE OF INVENTION: Recombinant Antigen for  
Diagnosing Rheumatoid  
Arthritis

(iii) NUMBER OF SEQUENCES: 3

## (iv) CORRESPONDENCE ADDRESS:

20

(A) ADDRESSEE: Thorpe, North &amp; Western

(B) STREET: 9035 South 700 East, Suite 200

(C) CITY: Sandy

(D) STATE: Utah

(E) COUNTRY: USA

25

(F) ZIP: 84070

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette, 3.5 inch, 720 Kb  
storage

30

(B) COMPUTER: IBM Thinkpad 340

(C) OPERATING SYSTEM: DOS 6.22

(D) SOFTWARE: Word Perfect 6.0

## (vi) CURRENT APPLICATION DATA:

35

(A) APPLICATION NUMBER: 08/364,081

(B) FILING DATE: 27-DEC-1994

(C) CLASSIFICATION:

## (vii) PRIOR APPLICATION DATA:

40

(A) APPLICATION NUMBER: 08/019,780

(B) FILING DATE: 19-FEB-1993

## (viii) ATTORNEY/AGENT INFORMATION:

45

(A) NAME: Alan J. Howarth

(B) REGISTRATION NUMBER: 36,553

(C) REFERENCE/DOCKET NUMBER: T781CIP

## (ix) TELECOMMUNICATION INFORMATION:

50

(A) TELEPHONE: (801)566-6633

(B) TELEFAX: (801)566-0750

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

55

(A) LENGTH: 13 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

60

(ii) MOLECULE TYPE: synthetic linker

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AATTCGCGGC CGC 13

10 (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 993 nucleotides

(B) TYPE: nucleic acid

15 (C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

20	ACT TCA GTT AAT TCT GCA GAA GCC AGC ACT AGT GCT AAC TCT GTA ACT 48
	Thr Ser Val Asn Ser Ala Glu Ala Ser Thr Ser Ala Asn Ser Val Thr
	1 5 10 15
25	TGT ACA TTT TCC CAT GGA TAT GAA AAG CCT GAA GAA TTG TGG ATC CCC 96
	Cys Thr Phe Ser His Gly Tyr Glu Lys Pro Glu Glu Leu Trp Ile Pro
	20 25 30
30	TTT TCT CCC GCA GCG AGT AGC TGC CAC AAT GCC AGT GGA AAG GTT GCA 144
	Phe Ser Pro Ala Ala Ser Ser Cys His Asn Ala Ser Gly Lys Glu Ala
	35 40 45
35	AAG GTT TGC ACC ATC AGT CCC TTG AGC TCC TTG ATT CCT GAA GCA GAA 192
	Lys Val Cys Thr Ile Ser Pro Leu Ser Ser Leu Ile Pro Glu Ala Glu
	50 55 60
40	GAT AGC TGG TGG ACG GGG GAT TCT GCT AGT CTC GAC ACG GCA GGC ATC 240
	Asp Ser Trp Trp Thr Gly Asp Ser Ala Ser Leu Asp Tyr Ala Gly Ile
	65 70 75 80
45	AAA CTC ACA GTT CCA ATC GAG AAG TTC CCC GTG ACA ACG GAG ACG TTT 288
	Lys Leu Thr Val Pro Ile Glu Lys Phe Pro Val Thr Thr Gln Thr Phe
	85 90 95
50	GTC GTC GGT TGC ATC AAG GGA GAG GAC GCA CAG AGT TGT ATG GTC ACG 336
	Val Val Gly Cys Ile Lys Gly Asp Asp Ala Gln Ser Cys Met Val Thr
	100 105 110
55	GTG ACA GTA CAA GCC AGA GCC TCA TCG GTC GTC AAT AAT GTC GCA AGG 384
	Val Thr Val Gln Ala Arg Ala Ser Val Val Asn Asn Val Ala Arg
	115 120 125
60	TGC TCC TAC GCT GCA GAC AGC ACT CTT GGT CCT GTC AAG TTC TCT GCG 432
	Cys Ser Tyr Gly Ala Asp Ser Thr Leu Gly Pro Val Lys Leu Ser Ala
	130 135 140
65	GAA GGA CCC ACT ACA ATG ACC CTC GTC TGC GGG AAA GAT GGA GTC AAA 480
	Glu Gly Pro Thr Thr Met Thr Leu Val Cys Gly Lys Asp Gly Val Lys
	145 150 155 160
70	GTT CCT CAA GAC AAC AAT CAG TAC TGT TCC GGG ACG ACG CTG ACT GGT 528
	Val Pro Gln Asp Asn Asn Gln Tyr Cys Ser Gly Thr Thr Leu Thr Gly
	165 170 175
75	TGC AAC GAG AAA TCG TTC AAA GAT ATT TTG CCA AAA TTA ACT GAG AAC 576
	Cys Asn Glu Lys Ser Phe Lys Asp Ile Leu Pro Lys Leu Thr Glu Asn
	180 185 190
80	CCG TCG CAG GGT AAC GCT TCG AGT GAT AAG GGT GCC ACG CTA ACG ATC 624
	Pro Trp Gln Gly Asn Ala Ser Ser Asp Lys Gly Ala Thr Leu Thr Ile
	195 200 205

20

5 AAG AAG GAA GCA TTT CCA GCC GAG TCA AAA AGC GTC ATT ATT GGA TGC 672  
 Lys Lys Glu Ala Phe Pro Ala Glu Ser Lys Ser Val Ile Ile Gly Cys  
 210 215 220

10 ACA GGG GGA TCG CCT GAG AAG CAT CAC TGT ACC GTG AAA CTG GAG TTT 720  
 Thr Gly Gly Ser Pro Glu Lys His His Cys Thr Val Lys Leu Glu Phe  
 225 230 235 240

15 GCC GGG GCT GCA GGG GGC GCC GGG GGT GGA CGA GGA GGA GCA GCC GGT 768  
 Ala Gly Ala Ala Gly Gly Ala Gly Gly Gly Gly Ala Ala Gly  
 245 250 255

20 GGA GCC GGG GGC GCC GCG GCT GCC GGC GGA GCA GGA GCA GGC GGA GGG 816  
 Gly Ala Gly Gly Ala Ala Ala Ala Gly Gly Ala Gly Ala Gly Gly  
 260 265 270

25 GCT GGT ACC GAC ACA GAT AAA TAT GTC ACA GGA ATA AAT GCC TCT CAT 864  
 Ala Gly Thr Asp Thr Asp Lys Tyr Val Thr Gly Asn Ala Ile Ser His  
 275 280 285

30 GGT CAG ACC ACT TAT GGT AAC GCT GAA GAC AAA GAG TAT CAG CAA GAA 912  
 Gly Gln Thr Thr Tyr Gly Asn Ala Glu Asp Lys Glu Tyr Gln Gln Glu  
 290 295 300

35 TTC GTG GGA ATT ATG ACA GTA ACT ATG ACA TTT AAA TTG GGG CCC CGT 960  
 Phe Val Gly Ile Met Thr Val Thr Met Thr Phe Lys Leu Gly Pro Arg  
 305 310 315 320

AAA GCT ACG GGA CGG TGG AAT CCT CAA CCT GGA 993  
 Lys Ala Thr Gly Arg Trp Asn Pro Gln Pro Gly  
 325 330

## (2) INFORMATION FOR SEQ ID NO:3:

## 40 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 331 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

## 45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Thr Ser Val Asn Ser Ala Glu Ala Ser Thr Ser Ala Asn Ser Val Thr  
 1 5 10 15

50 Cys Thr Phe Ser His Gly Tyr Glu Lys Pro Glu Glu Leu Trp Ile Pro  
 20 25 30

55 Phe Ser Pro Ala Ala Ser Ser Cys His Asn Ala Ser Gly Lys Glu Ala  
 35 40 45

60 Lys Val Cys Thr Ile Ser Pro Leu Ser Ser Leu Ile Pro Glu Ala Glu  
 50 55 60

65 Asp Ser Trp Trp Thr Gly Asp Ser Ala Ser Leu Asp Tyr Ala Gly Ile  
 65 70 75 80

70 Lys Leu Thr Val Pro Ile Glu Lys Phe Pro Val Thr Thr Gln Thr Phe  
 85 90 95

21

5	Val Val Gly Cys Ile Lys Gly Asp Asp Ala Gln Ser Cys Met Val Thr	100	105	110
10	Val Thr Val Gln Ala Arg Ala Ser Ser Val Val Asn Asn Val Ala Arg	115	120	125
15	Cys Ser Tyr Gly Ala Asp Ser Thr Leu Gly Pro Val Lys Leu Ser Ala	130	135	140
20	Glu Gly Pro Thr Thr Met Thr Leu Val Cys Gly Lys Asp Gly Val Lys	145	150	155
25	Val Pro Gln Asp Asn Asn Gln Tyr Cys Ser Gly Thr Thr Leu Thr Gly	165	170	175
30	Cys Asn Glu Lys Ser Phe Lys Asp Ile Leu Pro Lys Leu Thr Glu Asn	180	185	190
35	Pro Trp Gln Gly Asn Ala Ser Ser Asp Lys Gly Ala Thr Leu Thr Ile	195	200	205
40	Lys Lys Glu Ala Phe Pro Ala Glu Ser Lys Ser Val Ile Ile Gly Cys	210	215	220
45	Thr Gly Gly Ser Pro Glu Lys His His Cys Thr Val Lys Leu Glu Phe	225	230	235
50	Ala Gly Ala Ala Gly Gly Ala Gly Gly Gly Gly Gly Gly Ala Ala Gly	245	250	255
55	Gly Ala Gly Gly Ala Ala Ala Ala Gly Gly Ala Gly Ala Gly Gly Gly	260	265	270
60	Ala Gly Thr Asp Thr Asp Lys Tyr Val Thr Gly Asn Ala Ile Ser His	275	280	285
65	Gly Gln Thr Thr Tyr Gly Asn Ala Glu Asp Lys Glu Tyr Gln Gln Glu	290	295	300
	Phe Val Gly Ile Met Thr Val Thr Met Thr Phe Lys Leu Gly Pro Arg	305	310	315
	Lys Ala Thr Gly Arg Trp Asn Pro Gln Pro Gly	325	330	

5

Claims

I claim:

1. An antigen for diagnosing rheumatoid arthritis comprising a peptide having an amino acid sequence selected from the group consisting of SEQ ID NO:3 and sequences substantially homologous thereto, wherein said antigen is reactive with rheumatoid arthritis-associated antibodies.

2. The antigen of claim 1 wherein said peptide is produced by expression of a recombinant gene in a host cell.

3. The antigen of claim 2 wherein said host cell is a prokaryotic cell.

4. The antigen of claim 3 wherein said host cell is *E. coli*.

5. The antigen of claim 4 wherein said peptide has the amino acid sequence identified as SEQ ID NO:3.

6. The antigen of claim 2 wherein said host cell is a eukaryotic cell.

7. The antigen of claim 1 wherein said polypeptide is produced by chemical synthesis.

8. The antigen of claim 1 wherein said rheumatoid arthritis-associated antibodies are IgM antibodies.

9. A method for producing a recombinant antigen for diagnosing rheumatoid arthritis comprising a peptide having an amino acid sequence selected from the group consisting of SEQ ID NO:3 and sequences substantially homologous thereto, wherein said antigen is reactive with rheumatoid arthritis-associated antibodies, the method comprising the steps of:

(a) making a cDNA library from polyadenylated RNA purified from human cells, wherein said library is prepared by randomly cloning cDNA derived from said polyadenylated RNA in a cloning vector such that recombinant vectors are produced;



5 (b) selecting a recombinant vector that expresses a recombinant antigen detected by antibodies in serum from a patient with rheumatoid arthritis;

(c) transforming a host with the recombinant vector selected in step (b), and culturing said transformed host in a suitable nutrient medium so that said recombinant antigen is expressed; and thereafter

(d) isolating the recombinant antigen.

10 10. The method as in Claim 9 wherein step (c) further comprises growing the host to an optimum cell density and then inducing expression of the recombinant antigen.

15 11. The method as in Claim 9 wherein step (d) further comprises purifying recombinant antigen by affinity chromatography.

20 12. A purified and isolated DNA for use in securing expression in a host cell of a peptide having at least a part of the primary structural conformation and the antigenic activity of naturally-occurring RAMA protein; said DNA selected from the group consisting of:

25 (a) SEQ ID NO:2;

(b) DNA that hybridizes to SEQ ID NO:2 or fragments thereof; and

30 (c) DNA that, but for the degeneracy of the genetic code, would hybridize to the DNA defined in (a) and (b).

13. The purified and isolated DNA of claim 12 further comprising a vector adapted for transformation of a host.

35 14. The purified and isolated DNA of claim 13 wherein the vector is selected from the group consisting of plasmids, cosmids, phagemids, phages, and viruses.

15. The purified and isolated DNA of claim 14 wherein the vector is a plasmid.

40 16. The purified and isolated DNA of claim 15 wherein the host cell is *E. coli*.

5           17. The purified and isolated DNA of claim 16 wherein said DNA is SEQ ID NO:2.

          18. The purified and isolated DNA of claim 14 wherein the host is a eukaryotic cell.

10           19. A transformed host containing a DNA segment for use in securing expression in said transformed host of a peptide having at least a part of the primary structural conformation and the antigenic activity of naturally-occurring RAMA protein; said DNA selected from the group consisting of:

15           (a) SEQ ID NO:2;

          (b) DNA that hybridizes to SEQ ID NO:2 or fragments thereof; and

          (c) DNA that, but for the degeneracy of the genetic code, would hybridize to the DNA defined in (a) and (b).  
20

          20. The transformed host of claim 19 wherein said DNA segment is borne on a recombinant vector.

          21. The transformed host of claim 20 wherein the recombinant vector is selected from the group consisting of plasmids, cosmids, phagemids, phages, and viruses.  
25

          22. The transformed host of claim 21 wherein the recombinant vector is a plasmid.

          23. The transformed host of of claim 22 wherein the host cell is *E. coli*.

30           24. The purified and isolated DNA of claim 23 wherein said DNA is SEQ ID NO:2.

          25. The purified and isolated DNA of claim 20 wherein the host is a eukaryotic cell.

35           26. A method for producing a recombinant DNA encoding an antigen for diagnosing rheumatoid arthritis comprising a peptide having an amino acid sequence selected from the group consisting of SEQ ID NO:3 and sequences substantially homologous thereto, wherein said antigen is reactive with rheumatoid arthritis-associated antibodies, comprising:  
40

          (a) isolating polyadenylated RNA from human cells;

5           (b) making double-stranded cDNA with the isolated polyadenylated RNA as template therefor;

          (c) inserting the double-stranded cDNA into a cloning vector to produce a recombinant expression vector, said recombinant expression vector having the  
10       DNA encoding the antigen in correct phase for expression thereof;

          (d) transforming a host with the recombinant expression vector so that said antigen is expressed; and

          (e) selecting the transformed host by detection  
15       with antibodies from serum of a rheumatoid arthritis patient wherein said antibodies bind said expressed antigen.

          27. The method as in Claim 26 wherein, in (e), transformed hosts having bound antibodies are detected  
20       with an anti-human IgM antibody-alkaline phosphatase conjugate and thereafter adding one or more alkaline phosphatase color development substrates.

          28. The method as in Claim 27 wherein the color development substrates comprise nitro blue tetrazolium  
25       and 5-bromo-4-chloro-3-indolyl phosphate.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/16558**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :C07K 14/435; C12N 1/21, 5/10, 15/12, 15/63; C12P 19/34, 21/02

US CL :435/69.1, 91.51, 240.2, 252.33, 320.1; 530/350; 536/23.5

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 91.51, 240.2, 252.33, 320.1; 530/350; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog

search terms: rheumatoid arthritis, RA, RAMA, antigen??, immunoglobulin, IgM, antibody or antibodies, immunoassay

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	American Journal of Clinical Pathology, Vol. 74, No. 6, issued December 1980, Halbert et al., "A quantitative enzyme immunoassay for IgM rheumatoid factor using human immunoglobulin G as substrate", pages 776-784, see the entire document.	1-28
A	Journal of Immunology, Vol. 141, No. 10, issued 15 November 1988, Burg et al., "Molecular analysis of the gene encoding the major surface antigen of Toxoplasma gondii", pages 3584-3591, see the entire document.	1-28
A	Journal of Immunology, Vol. 146, No. 2, issued 15 January 1991, Artandi et al., "Molecular analysis of IgM rheumatoid factor binding to chimeric IgG", pages 603-610, see the entire document.	1-28



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A document defining the general state of the art which is not considered to be of particular relevance	*X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E earlier document published on or after the international filing date	*Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G	document member of the same patent family
*O document referring to an oral disclosure, use, exhibition or other means		
*P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

04 APRIL 1996

Date of mailing of the international search report

18 APR 1996

Name and mailing address of the ISA/US  
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1/1

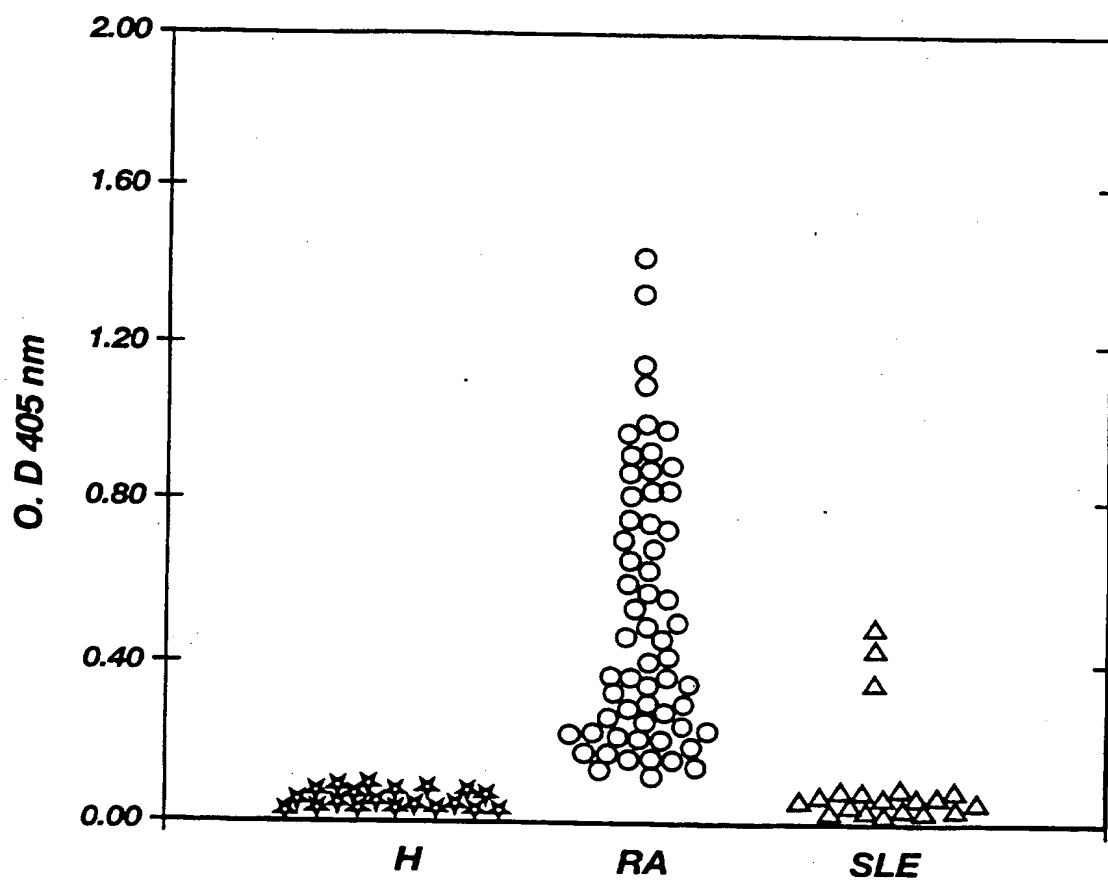


Fig. 1